(54) Title: USE OF AN OSMOLYTE FOR TREATING THE EFFECTS OF AN INFECTION, AN INFLAMMATION OR AN IMMUNE DYSFUNCTION

(57) Abstract

    The present invention is directed to a therapy and suitable compositions involving an effective amount of an osmolyte capable of treating the effects of an infection, an inflammation or an immune dysfunction.

USE OF AN OSMOLYTE FOR TREATING THE EFFECTS OF AN INFECTION, AN INFLAMMATION OR AN IMMUNE DYSFUNCTION.

Field of invention

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The present invention relates to the use of osmolytes in the manufacture of therapeutic agent for treating the effects of an infection, inflammation or for treating an immune dysfunction.

10 **Background of the invention**

Liver macrophages or Kupffer cells belong to the mononuclear phagocyte system and play an important role in the body's defense machinery, see e.g. Eur J Biochem, 1990, Vol. 192, pag. 245-261; K Decker. The Kupffer cells are the major

15 producer of eicosanoids, such as prostaglandin E2 , D2 and thromboxane A2 in the liver. The eicosanoid production by the Kupffer cells plays a major role in the pathogenesis of septic shock and may contribute to liver cell damages under these conditions. The formation of eicosanoids is dependent on enzymatic conversion of arachidonic acid by cyclooxygenase (Cox) which appears in two iso-forms. One form (Cox-1) is constitutively

20 expressed, whereas one form (Cox-2) is induced in macrophages upon the response to proinflammatory stimuli.

It has been demonstrated in, Biochem. J, 1995, Vol. 312, page 135-142, F Zhang et al., that endotoxin enhances the expression of inducible cyclooxygenase-2 (Cox-2), with the result of an increased formation of prostanoids by Kupffer cells and other

25 macrophages. It was also demonstrated in this publication that a similar prostaglandin E2, D2 and thromboxane B2 formation and Cox-2-expression is stimulated about 7-10-fold, when the ambient osmolarity increases from 300 to 350 mosmol/l.

The remarkably sensitive and potent osmoregulation of Kupffer cell function suggests that cell volume homeostasis is a critical factor for Kupffer cell function. It would

30 therefore be desirable to investigate the activity of organic osmolytes of the type normally

used by the cells for adaptation to osmolality changes by accumulation or release in response to hyperosmotic cell shrinkage and hyperosmotic cell swelling, respectively, in order to maintain cell volume homeostasis. This type of organic osmolytes need to be non-perturbing solutes that do not interfere with protein function even when occurring at high

5   intracellular concentrations. Such a prerequisite may explain why only a few classes of organic compounds, viz. polyols (e.g. inositol and sorbitol), methylamines (betaine, α-glycerophosphorylcholine) and certain amino acids such as taurine have evolved as osmolytes in living cells.

In mammals, osmolytes have been identified in astrocytes, renal medulla cells

10   and lens epithelia. The need for osmolytes in renal medulla cells is obvious, because ambient medullary osmolarity can increase up to 3800 mosmol/l during antidiuresis and decrease to 170 mosmol/l during diuresis. In the antidiuretic state (high extracellular osmolarity), intracellular osmolarity increase in renal medullary cells as the result of the intracellular accumulation of inositol and betaine, which are taken up via Na+-dependent

15   transporters. These Na+-dependent transporters are induced upon hyperosmotic exposure in renal cells and astrocytes. Recent studies with Madine-Darby canine kidney (MDCK) cells have identified a hypertonic stress-responsive element in the 5'-flanking region of the mammalian BGT-1 gene (betaine transporter).

In a parallel study disclosed in FEBS Letters, 1995, Vol. 377, pages 47-50,

20   U Warskulate et al., betaine is identified as an osmolyte in mouse macrophages. The betaine uptake in mouse macrophages was significantly stimulated when the cells were exposed to a hyperosmotic (450 mosm/l) medium. From the results of this study it was concluded that betaine availability could be a potential site for the regulation of macrophage cell function.

25   Certain organic osmolytes have previously been suggested in the International Patent Application WO 91/14435 as supplements to protect cells in a dehydrated environment from volume changes. Also in Biochem. Journal, 1992, Vol. 282, pages 69-73, it is demonstrated that SV-3T3 cells (fibroblasts) subjected to hyperosmotic conditions may retain normal function in terms of rate of cell proliferation and protein

30   synthesis in the presence of an osmolyte. Even if these publications may consider a

therapeutic utility of certain osmolytes, there are no disclosures of how osmolytes can affect cells which mediates pathological events, specifically immune competent cells both at hyperosmolar conditions and in conditions with normal osmolarity.

According to the present invention, it has been surprisingly found that certain organic osmolytes, such as betaine, have a powerful capacity, besides restoring the intracellular osmolality to ambient levels, to affect cellular functions which are parts of the mechanisms of inflammation, infection and immune dysfunction. For example, the present invention shows a suppression of the levels of cyclooxygenase-2 and thereby also the production of eicosanoids, such as prostaglandin E2, produced following endotoxin challenge. These findings exemplify that otherwise metabolically inert osmolytes exert a regulatory effect on the inflammatory response of immune competent cells. Furthermore, it renders such osmolytes a pharmacological potency comparable to conventional anti-inflammatory therapy, such as non-steroidal anti-inflammatory drugs (NSAID:s), but without the well-disclosed side-effects of those compounds.

Description of the invention

It is the object of the present invention to use a therapeutically effective amount of at least one osmolyte in the preparation of a therapeutic agent capable of treating the effects of an infection, an inflammation or an immune dysfunction.

It is also the object of the present invention to provide compositions which enable the treatment or prevention of an infection, an inflammation or an immune dysfunction, for example by a therapeutically effective supplementation of at least one osmolyte to a parenteral nutrient which is modified to facilitate cellular osmolyte uptake.

The osmolytes being used according to the present invention are particularly aimed to affect cells which have an active part in producing mediators of said complications or have an active part in the immune system. Such cells typically include, but are not limited to, immune competent cells, endothelial cells and hepatocytes and are examplified below by macrophages, Kupffer cells, and liver sinusoidal endothelial cells. The effects originating from infection, inflammation and immune dysfunction which are

treatable by the utility of the present invention include, but are not limited to, an increase

in the cellular cyclooxygenase activity, an increase in the cellular inducible nitric oxide

synthase levels, raised TNF-levels as typically triggered by bacterial endotoxins, cytokines,

microorganisms or their fragments or products, other inflammatory mediators appearing as

5   a result of inflammatory conditions, the transition to neoplastic cells, other dysfunctions of

the immune system, or from tissue injuries. These effects can be followed by cell death in

vital organs, such as programmed cell death (apoptosis) or necrosis which consequently

also can be treated by inventive osmolyte therapy. The mentioned effects can also be

accompanied by cellular volume changes induced by plasma hyperosmolarity, for example

10   in severe infectious diarrhea where extensive fluid losses are elicited and in other fluid

imbalance conditions where a fluid therapy is mandatory.

       According to the present invention osmolytes are defined as agents that can

be used by the cells for regulation of the cell hydration by a specific transport mechanism

through the cellular membranes. Such agents have traditionally not been considered to

15   intervene to alter the cellular metabolism, except for their function as substrates in

metabolic pathways. The osmolytes used according to the present invention are preferably

selected from a group of organic osmolytes consisting of polyols, amino acids and

methylamines that will not interfere with the cellular protein function, although being

present at high intracellular concentrations. Preferred osmolytes are polyols, such as myo-

20   inositol and sorbitol, methylamines, such as betaine and alpha-glycerolphosphorylcholine

and certain amino acids, such as taurine. Also salts and precursors of such osmolytes are

conceivable to use in the present invention, such as alkyl esters of suitable osmolytes and

oligopeptide derivatives According to the present invention chemical modifications of

osmolytes are conceivable in order to obtain osmolytic derivatives with facilitated

25   transmembrane transport and thereby increase their intracellular uptake. Other objectives

for chemical modifications can be to introduce conjugates of osmolytes with improved

target seeking and capacity, to obtain a desired solubility, polarity or physical stability.

       It is especially preferable to use inositols, amino acids and methylamines,

and particularly preferable to use taurine, betaine and myo-inositol, their salts and

30   precursors such as functional derivatives of betaine or taurine or releasing conjugates, for

example alkyl esters of betaine. Also biological precursors of such compounds are considered as part of the present invention. In particular, choline is desirable to select considering its capacity to be conversed to betaine in certain cell types, such as hepatocytes, for transportation to betaine deficient cells without such capacity, such as

5　Kupffer cells. It is to be understood that also the use of mixtures of the mentioned osmolytes can be a part of the present invention, for example mixtures of amino acids and methylamines with osmolytic capacity. A preferred combination of osmolytes is taurine and betaine in effective amounts.

In clinical practice, the present invention provides for a new use of osmolytes

10　in therapy and provides new compositions together with selected nutrients. It is obvious that the present invention is applicable in the treatment of reducing the effects of infection, inflammation and immune dysfunction in any pathological condition which involves osmotic stress on cells or tissues or in conditions were osmolyte supply or synthesis is inadequate. Such conditions involving osmotic stress can result from fluid losses, for

15　example from diarrhea, burns and sepsis, or be the result of increased levels of circulating metabolites, for example urea and glucose. Increased urea levels can appear as a consequence of increased protein catabolism, e.g. post trauma, or from impaired nitrogen metabolism including the state of uremia. An increase in circulating levels of glucose is a well recognized complication in diabetes as well as reversible insulin resistance, in the

20　catabolic state following trauma and during the progress of certain cancer forms. Furthermore the use of osmolytes according to the present invention would also be useful in the treatment of colon cancer involving an increase in cyclooxygenase levels where conventionally non-steroidal antiinflammatoric drugs are administered to suppress the carcinogenity.

25　Conventionally used parenteral nutrition products, as required by many patients suffering from the above-mentioned complications, generally are low or even completely deficient in betaine and its precursor choline. The betaine deficiency facilitates an augmented eicosanoid production which is known to precipitate cholestasis, a well recognized inflammatory complication in the bile ducts which might appear during

30　parenteral nutrition. For this reason, it is also an important aspect of the present invention

to provide such parenteral nutritional products with an effective amount of the mentioned osmolytes, in order to provide nutritional regimens which aim to prevent chlestasis. According to one embodiment of the present invention, the osmolyte is used together with a parenteral or enteral nutrient solution deficient compounds interfering with the osmolyte uptake in immune competent cells. Examples of such nutrients are fluid sources of amino acids comprising amino acids or conjugates thereof (e.g. peptides) or amino acid precursors, lipid emulsions comprising certain beneficial long-chain fatty acids and/or medium chain fatty acids as energy suppliers and sugars or carbohydrates. Such compositions are selected to effectively combine osmolyte therapy with an appropriate nutritional therapy suitably adapted in its composition to patients suffering from severe infections or inflammatory conditions or impairments of the immune system. Such diets are well-known and can be made available for enteral or parenteral administration and the skilled practitioner can readily adapt them to be administered together with an osmolyte by removing certain amino acids (or other compounds) which may interfere with the cellular osmolyte uptake. When the osmolytes are selected among amino acids and methylamines it is preferred that the nutrients are deficient in certain amino acids interfering with the cellular uptake of osmolytes. If the osmolyte specifically is a betaine (or a salt or precursor thereof) it is preferred that a composition is deficient in alanine and proline, which means that it either lacks these amino acids or that it has a suitably low level of them in order to minimize their interference with the cellular osmolyte uptake. According to another embodiment of the present invention, the osmolyte is selected among methylamines and amino acids with osmolytic capacity formulated in an enteral or parenteral composition optionally together with one or several nutrients selected from a fluid amino acid source, a lipid emulsion and carbohydrates. This composition can further comprise such constituents conventionally used in nutrition, such as vitamins, trace elements, electrolytes, as well as drugs suitable to administer to a patient in a specific clinic situation, such as antibiotics and anesthetics. It is especially preferred that the composition comprises such an amount of the osmolytes so a plasma concentration within the approximate range of 0.01 to 10 mM and a preferred range 0.1 to 1-2 mM is obtained after its administration. The most preferred

osmolytes according to the present invention are betaine and taurine and their salts or

conjugates (functional equivalents) which can be used either alone or together.

The following part aims to exemplify the present invention and shall not be regarded as

limiting for the scope of invention.

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Detailed and exemplifying description of the invention

Fig. 1 shows time-dependent induction of BGT-1 (betaine transport protein)

and Cox-2 mRNA levels in Kupffer cells during hyperosmolarity. The Kupffer cells were

10   exposed to LPS (1 μg/ml) in normoosmotic (305 mosmol/l) or hyperosmotic (405

mosmol/l) media for the time periods indicated and mRNA levels for BGT-1,

cyclooxygenase-2 (Cox-2) and glyceraldehydephosphate dehydrogenase (GAPDH) were

determined by Northern blot analysis.

Fig. 2 demonstrates the induction of BGT-1 mRNA by hyperosmolarity in

15   unstimulated Kupffer cells. Rat Kupffer cells were exposed for 12 h in hypoosmotic (205

mosmol/l), hyperosmotic (405 mosmol/l) or normoosmotic media (305 mosmol/l) and the

mRNA levels for BGT-1 and GAPDH were determined by Northern blot analysis.

Fig. 3 displays the effect of medium osmolarity on the mRNA levels for

BGT-1, Cox-1, Cox-2 and GAPDH. LPS stimulated rat Kupffer cells were exposed for 12

20   h to media with the osmolarity indicated. Osmolarity changes were performed by

appropriate addition/removal of NaCl. The mRNA levels for BGT-1 and GAPDH were

determined by Northern blot analysis. This experiment is representative of three separate

experiments.

Fig. 4 shows the effects of betaine, taurine and glutamine on BGT-1 mRNA

25   levels in Kupffer cells. Rat Kupffer cells were exposed to hyperosmotic medium (380

mosmol/l) for 12 h. This medium contained no further additions (control) or betaine,

taurine or glutamine at the concentrations indicated. The mRNA levels for BGT-1 and

GAPDH were determined by Northern blot analysis. This experiment is representation of 3

different experiments.

Fig. 5 shows the effect of betaine on the hyperosmolarity-induced induction of Cox-2 protein (A) and mRNA (B) in LPS-stimulated Kupffer cells.(A) Kupffer cells were exposed to LPS (1 µg/ml) in normoosmotic (305 mosmol/l) or hyperosmotic (405 mosmol/l) media for 24h in the absence or in the presence of betaine as indicated. Then

5   whole cell extracts were used for Western blot analysis as described in Methods. (B) Kupffer cells were treated to LPS in normoosmotic or hyperosmotic media with the indicated concentrations of betaine for 12 h. The mRNA levels for BGT-1 and GAPDH were determined by Northern blot analysis.

Fig. 6 shows the time-dependent induction of BGT-1 (betaine transporting

10   protein) and TAUT (taurine transporting protein) and SMIT (the myo-inositol transporter) mRNA-levels in rat Kupffer cells. The Kupffer cells were exposed to LPS (1 µg/ml) in normoosmotic (305 mosmol/l) or hyperosmotic (405 mosmol/l) media for the time periods indicated and mRNA levels for BGT-1, SMIT, TAUT and glyceraldehydephosphate dehydrogenase (GAPDH) as a standard were determined by Northern blot analysis.

15   Fig. 7 shows the influence of myo-inositol, taurine and betaine on cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mRNA levels in rat sinusoidal cells during hyperosmolarity. Cultured sinusoidal cells were exposed to LPS (lipopolysaccharide 1 µg/ml to produce an endotoxic effect) for 6 h in normoosmotic (305 mosmol/l) or hyperosmotic (405 mosmol/l) in the presence or absence of 5 mmol/l of

20   myoinositol, taurine or betaine). mRNA levels of COX-2 and iNOS were determined by Northern blot analysis (7 µg of total RNA per lane). Glyceraldehyde -3-phosphatase mRNA was used for standardization.

Fig. 8 shows the effect of ambient osmolality on mRNA levels for the betaine transporter (BGT-1), the taurine transporter (TAUT), the myo-inositol transporter

25   (SMIT) and GAPDH in the rat liver endothelial cells. Changes in osmolality were performed by appropriate addition/removal of sodium chloride. The mRNA levels were determined by Northern blot analysis.

Fig. 9 shows the osmolarity dependent induction of the betaine transporter (BGT-1), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and GAPDH

30   (as a reference) in rat Kupffer cells following exposure to LPS (1 µg/ml) in normoosmotic

media (305 mosmol/l) or hyperosmotic media (375 mosmol/l). the mRNA levels were determined by Northern blot analysis.

Fig. 10 shows the influence of betaine on the transporters for betaine and taurine (BGT-1 and TAUT) and on inducible nitric oxide synthase mRNA levels in RAW

5   264.7 mouse macrophages during hyperosmolarity. The macrophages were exposed to LPS (1 μg/ml) for 6 hours in the presence or absence of 0.1 or 5 mmol/l betaine. The mRNA levels of the transporters and iNOS were determined by Northern blot analysis.

Fig. 11A shows the modulation of the CD95 ligand mRNA expression (a mediator for apoptosis) in rat Kupffer cells in response to LPS challenge (1 ug/ml for 6h).

10   In experiments shown in bars 1 and 2, the cells were not incubated with LPS. In experiments shown in bars 2 and 4, 5 mmol/l betaine was added 30 min before and throughout the whole 6 h measurement period. Total RNA was extracted, reverse transcribed and quantified by using PCR technique. Results are expressed as the ratio of number of CD95 ligand transcripts obtained with the indicated primers to the numbers of

15   rat hypoxanthine-guanine phsophoribyltransferase (HPRT) transcripts.

Fig. 11 B shows the same experiment as in Fig 11 A performed with rat sinusoidal endothelial cells.

Table 1 shows how the hyperosmolarity induced betaine uptake is affected by the presence

20   of various amino acids.

Table 2 shows the effect of betaine on the prostaglandin E2 production by lipopolysaccharide stimulated rat Kupffer cells in normosmotic and hyperosmotic medium, respectively.

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Material and methods

Culture medium RPMI 1640 (without phenol red) and fetal calf serum (FCS) were from Biochrom (Berlin, Germany), oligonucleotide-labelling kit were from Pharmacia (Freiburg,

30   Germany). Guanidine thiocyanate and sodium lauroylsarcosinate were from Fluka

(Karlsruhe, Germany). (α-32P)dCTP (3000 Ci/mmol) and Hybond-N nylon membranes
were purchased from Amersham Buchler (Braunschweig, Germany). A plasmid containing
full-length BGT-1 cDNA was kindly provided by Dr. H. Moo Kwon (Division of
Nephrology, The John Hopkins University School of Medicine, Baltimore, MD, U.S.A.).

5   The cyclooxygenase (Cox-1 and Cox-2) cDNA probes were from Cayman Chemical
Company (Ann Arbor, Michigan) and the 1.0 Kb cDNA fragment for glyceraldehyde-3-
phosphate dehydrogenase (GAPDH) was used for standardization was from Clontech
(Palo Alto, U.S.A.).

10   <u>Isolation and culture of Kupffer cells</u>

Kupffer cells from male Wistar rats of 300-400 g body weight raised in the local institute
for laboratory animals were isolated by collagenase-pronase perfusion and separated by a
single Nycodenz gradient and centrifugal elutriation. Cells were cultured in RPMI 1640

15   medium supplemented with 10% heat-inactivated fetal calf serum (FCS) for 48 h. The
experiments were performed during the following 24 h using Krebs-Henseleit hydrogen
carbonate buffer (pH 7.4) containing 10 mM glucose and 1% FCS. At that time the
cultures consisted of more than 99% Kupffer cells as demonstrated by their morphological
appearance and their ability to phagocytose 1 μm Latex particles, which is not observed in

20   cultured endothelial cells. The osmolarity was varied by changing the NaCl concentration.
The viability of Kupffer cells was more than 95% as assessed by trypan blue exclusion.
Kupffer cell volume was measured by flow resistance cytometry using a Casy 1 cell
counter and analyzer system (Schärfe Systeme, Reutlingen, Germany). In normoosmotic
medium, the average Kupffer cell volume was 724 ± 24 fl (7 different preparations).

25   Protein content was 0.039 ± 0.009 mg per 106 cells (n=7). Assuming a water content of
80% of whole Kupffer cell volume, a mean intracellular water space of 14,9 μl/mg protein
is estimated. Viability of the incubations was routinely tested by lactate dehydrogenase
(LDH) release at the end of the incubation. 12-24 h hyperosmotic (405 mosmol/l) or a
hypoosmotic (205 mosmol/l) exposure was without effect on LDH release.

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Determination of PGE2

Kupffer cell supernatants were assayed for PGE2 by competitive binding
radioimmunoassay (RIA) using [3H]labeled PGE2 (Amersham, Braunschweig, Germany)
5   and a specific antiserum to PGE2 (Sigma, Deisenhofen, Germany). For further details, see
Biochem. J, 1995, Vol. 312, page 135-142, F Zhang et al.
PGE2 is considered as representative in this context as a general marker for the
eicosanoids affected by the osmolyte therapy

10   Western blotting

Kupffer cells were washed with phosphate-buffered saline, and were lysed in 100 mM
NaCl containing 10 mM-Tris/HCl (pH 7.3), 2 mM EDTA, 0.5% deoxycholate, 1%
Nonidet P40, 10 mM MgCl2, 1 mM phenylmethanesulphonyl flouoride, and 10 µg of
15   aprotinin/ml for 10 min on ice. Lysates containing 30 µg of protein were mixed with an
equal volume of Laemmli sample buffer, and denatured by boiling for 5 min. After
SDS/PAGE (10% gel) and electrophoretic transfer, the nitrocellulose filters were blocked
using 3% defatted dried milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h.
Filters were incubated overnight with a specific antibody to Cox-2 (Cayman chemicals,
20   Ann Arbor, MI) used at a dilution of 1:1000. After washing in TBS-T, the filters were
incubated with horseradish peroxidase-conjugated anti-rabbit antibody, again washed four
times in TBS-T and exposed to enhanced chemiluminescence reagents for 1 min. Blots
were exposed to Kodax SAR-5 film for 1-5 min.

25   Northern blot analysis

Total RNA from near-confluent culture plates of Kupffer cells was isolated by using
guanidinethiocyanate solution. RNA samples were electrophoresed in a 0.8% agarose/3%
formaldehyde and then blotted onto Hybond-N nylon membranes with 20X SSC (3 M
30   NaCl, 0.3 M sodium citrate). After brief rinsing with water and UV-crosslinking (Hoefer

UV-crosslinker 500), the membranes were inspected under UV illumination to determine
RNA integrity and location of the 28S and 18S rRNA bands. Blots were then subjected to
a 3 h-prehybridization at 43¡C in 50% deionized formamide, in sodium phosphate buffer
(0.25 M, pH 7.2), containing 0.25 M NaCl, 1 mM EDTA, 100 mg/ml salmon sperm DNA
5   and 7% SDS. Hybridization was carried out in the same solution with approx. 106 cpm/ml
(α-32P)dCTP-labeled random primed BGT1, Cox-1 or Cox-2 and GAPDH cDNA probes.
Membranes were washed three times in 2x SSC/0.1% SDS and twice in sodium phosphate
buffer (25 mM, pH 7.2)/EDTA (1 mM)/1 % SDS. Blots were then exposed to Kodak AR
X-omat film at 70°C with intensifying screens and analysed with PDI densitometry
10   scanning (Pharmacia, Freiburg, Germany).

Statistics

Values are expressed as mean S.E.M (n= number of Kupffer cell preparations). Statistical
15   analysis was performed using Student's t-test. p<0.05 was considered to be statistically
significant.

Discussion of the results

20   As shown in Fig. 1, hyperosmotic exposure of Kupffer cells led to a strong
and time dependent increase in BGT-1 mRNA levels. Maximal BGT-1 mRNA levels were
found after 12 h of hyperosmotic exposure (Fig. 1). The time course of hyperosmolarity-
stimulated expression of BGT1 mRNA roughly paralleled the increase in mRNA levels for
cyclooxygenase-2 (Cox-2), when Kupffer cells were simultaneously exposed to
25   lipopolysaccharide (LPS) (Fig. 1).
              In the absence of lipopolysaccharide, hyperosmolarity likewise induced
BGT-1 mRNA (Fig. 2); under these conditions induction of Cox-2 mRNA is not observed.
              In the presence of LPS, BGT-1 and Cox-2 mRNA levels, but not the mRNA
levels for cyclooxygenase-1 (Cox-1) were strongly dependent upon the ambient osmolarity
30   (Fig. 3). As shown in Fig. 4 and Fig. 5, the hyperosmolarity-induced induction of BGT-1

mRNA was counteracted by betaine in a concentration-dependent manner, but not by taurine or glutamine. This suggests an important function of betaine among osmolytes in the regulation of immune competent cells. As previously shown in Biochem. J, 1995, Vol. 312. page 135-142, F Zhang et al., hyperosmotic exposure of LPS-stimulated Kupffer

5   cells markedly increases prostaglandin E2 (PGE2) formation due to an induction of cyclooxygenase-2 (Cox-2). However, when betaine is added to treat the immune competent cells (Kupffer cells) according to the present invention, the hyperosmolarity-induced stimulation of PGE2 production is significantly diminished (Table 2). As shown in Fig 5, betaine prevented the hyperosmolarity/LPS-induced induction of cyclooxygenase-2

10   at the level of both, immunoreactive protein (Fig. 5A) and mRNA (Fig. 5B).

Functional organic osmolytes besides betaine and taurine in Kupffer cell are not yet clear. However, neither taurine (see Fig. 4) nor myo-inositol (see Table 1) had any significant effect on the hyperosmotic-induction of the betaine transport. It is possible that other forms of immune competent cells than Kupffer cells could be shown to be dependent

15   on other osmolytes for their regulation of their immune function.

It is demonstrated in Fig. 6 that mRNA levels for both betaine transporting protein (BGT-1) and taurine transporting protein (TAUT) were expressed in Kupffer cells and were strongly dependent on ambient osmolarity. Hence it is shown that both betaine and taurine are important factors for the regulation of the cellular function in immune

20   competent cells. Besides the transporters for betaine and taurine, also the myo-inositol transporter (SMIT) is expressed in Kupffer cells and in liver endothelial cells (see Fig. 8). Consequently, these experiments demonstrate betaine, taurine and myo-inositol as therapeutically effective osmolytes. There is a time dependent increase in BGT-1 and TAUT and SMIT mRNA expression in response to hyperosmolarity (Fig. 6) while the

25   expression of the osmolyte transporters increase with increased osmolality (Fig. 8). These findings indicate that the therapeutic efficacy can be optimized in relation to the targeted cell type and the timing of the intervention.

The findings demonstrated in Fig. 7 indicate that the osmolytes myo-inositol, taurine and betaine are effective in downregulating COX-2 expression also in endothelial

30   cells.

The results shown in Fig. 9 demonstrate that in Kupffer cells, osmolytes are effective in downregulating COX-2 and iNOS expression also at normoosmolar conditions which implies that osmolytes exert these effects through a mechanism independent of cellular hydration.

5   The results of Fig. 10 show that osmolytes are effective in downregulating inducible nitric oxide synthase (iNOS) in macrophages. As iNOS is mediator in complications following inflammation and infection these results further support the utility of selected osmolytes as therapeutically effective agents for treating the effects of such complications. Fig. 11 A and 11 B demonstrates the capacity of osmolytes

10   in protection of apoptosis.

As shown in Table 1, hyperosmolarity-stimulated betaine uptake was strongly inhibited by 10 mmol/l concentrations of proline and gamma-aminobutyric acid and to a lesser extent by alanine, histidine and methylaminoisobutyrate, whereas taurine and choline were less effective. In both, kidney and Kupffer cells, BGT-1 mRNA levels

15   increase in response to hyperosmolarity and the induced betaine transport activity is Na- and Cl-dependent and is inhibited by GABA and proline. In kidney, betaine and myo-inositol counteract the hyperosmolarity-induced induction of their respective transporters, such a behavior is also found for the betaine transporter in Kupffer cells, see Table 1. The findings in Biochem. J, 1995, Vol. 312, page 135-142, F Zhang et al., have indicated

20   that eicosanoid formation by LPS-stimulated Kupffer cells is critically dependent upon the ambient osmolarity within a narrow range. Prostaglandin E2 formation increases up to 10-fold in response to hyperosmotic exposures, see Table 2 and Fig. 3, due to an induction of Cox-2.

Table 2 and Fig. 5 also demonstrates that betaine suppresses the

25   hyperosmolarity-induced increase in PGE2 formation and Cox-2 induction, thus indicating that this osmolyte does interfere with the inflammatory response of immune competent cells, such as liver macrophages. An addition of betaine (1 mmol/l) to hyperosmotically exposed Kupffer cells abolished the strong induction of Cox-2 and the increase of prostaglandin E2 formation. The betaine concentrations required for such an effect (Table

30   2) are well in the range of the physiological plasma concentration, which is reported to be

20-120 µmol/l. It should be stressed that the findings of Table 2 demonstrate that

osmolytes, and in particular betaine, have a capacity of decreasing Cox-2 levels induced by

endotoxins or by other mediators or effectors of the immune system, both in

normoosmolar and in hyperosmolar media. A therapeutic supply of selected organic,

5   endogenous osmolytes thus has a considerable potential in the treatment of infections,

inflammatory conditions and immune dysfunctions, where it could be of advantage to

reduce the induced inflammatory response of eicosanoids by a reduction of Cox-2 levels or

to restore the normal function of immune competent cells. Furthermore, the findings in

Table 2, related to the downregulation of the inflammatory process, also in cases without

10   osmotic stress, makes it obvious to conclude that certain osmolytes can alleviate

inflammation states in general. For example could inflammatory bowel syndromes

potentially be treated with such osmolytes by enteral or parenteral administration.

Table 1:

15

Inhibition of hyperosmolarity-induced betaine uptake by various amino acids.

Kupffer cells were exposed for 12 h to hyperosmotic medium (405 mosmol/l); thereafter

[14C] betaine (10 µmol/l) uptake was measured in the absence (control) or presence of

20   various effectors, which were added at a concentration of 10 mmol/l, each. Data are give

as mean ±SEM and are from four different experiments.

*significantly different from the control (p<0.05).

	Betaine uptake (nmol/mg protein/2h)	% inhibition of betaine uptake
Control	17.9±1.1	0
GABA	3.5±0.7 *	80
L-proline	1.2±0.1 *	93
MeAIB	8.4±1.2 *	53
L-alanine	10.8±1.2 *	40
L-histidine	7.8±0.9 *	57
choline	16.4±1.4	9
taurine	18.4±1.4	0
myo-inositol	18.5±1.3	0

Claims

1. Use of an effective amount of at least one osmolyte in the preparation of a therapeutic agent capable of preventing or treating the effects of an infection, an inflammation or an
5   immune dysfunction.

2. Use according to claim 1, wherein the osmolyte exerts its therapeutic effects by affecting cells producing mediators of said complications.

10   3. Use according to claim 2, wherein said cells are selected among immune competent cells, endothelial cells and hepatocytes.

4. Use according to any of claims 1 to 3, wherein said effects involves an increase in the activity of cyclooxygenase (Cox) and/or inducible nitric oxide synthase (iNOS) and/or
15   cell death.

5. Use according to any of claims 1 to 4, wherein said effects involves hyperosmolarity.

6. Use according to claims 1 to 5 **characterized in that** the osmolyte is organic and
20   selected from a group consisting of polyols, amino acids and methylamines.

7. Use according to claim 4 **characterized in that** said osmolyte is selected from a group consisting of betaine, taurine and myoinositol, their salts or precursors.

25   8. Use according to any of claims 1 to 7, wherein the osmolyte containing preparation osmolyte is a parenteral nutrient deficient in agents interfering with the cellular osmolyte uptake.

9. Use according to claim 8, wherein the osmolyte is betaine its salts or precursors and the
30   nutrient is deficient in the amino acids alanine and proline.

Table 2:

Effect of betaine on prostaglandin E2 production by LPS-stimulated rat Kupffer cells

Rat Kupffer cells were incubated with LPS (1 µg/ml) in normoosmotic (305 mosmol/l) or hyperosmotic (380 mosmol/l) medium for 24 h. PGE2 formation was measured by radioimmunoassay. Data are given as mean ±SEM and are from four different experiments.

*significantly different from the control (p<0.05).

	PGE2 (pmol/106/24h)	
	305 mosmol/l	380 mosmol/l
Control	24.7±2.9	185.8±24
Betaine 0.1mM	20.1±4.2	104.1±28 *
Betaine 1mM	16.1±4.4	35.6±5.8 *
Betaine 5mM	13.6±3.9	21.3±2.5 *
Betaine 10mM	11.8±3.5 *	24.3±3.3 *

10. Use of an effective amount of an osmolyte selected among betaine, taurine, myoinositol, their salts or precursors, in the preparation of a therapeutic agent for treating a patient having, or being at risk of acquiring, complications involving cellular volume changes in immune competent cells or endothelial cells.

11. A composition comprising a therapeutically effective amount of at least one osmolyte and least one nutrient selected from a group consisting of lipid emulsions, fluid sources of amino acids and carbohydrates **characterized in that** said nutrient is deficient in nutritionally useful compounds interfering with the osmolyte uptake.

12. A composition according to claims 11 **characterized in that** the osmolyte is selected from a group consisting of polyols, amino acids and methylamines.

13. A composition according to claim 12 **characterized in that** said osmolyte is selected from a group consisting of betaine, taurine and myo-inosiol, their salts and precursors.

14. A composition according to claims 12 or 13, wherein said nutrient is deficient in amino acids interfering with the osmolyte uptake.

15. A composition according to claims 14 comprising betaine or its salts or conjugates, wherein said nutrient is deficient in the amino acids proline and alanine.

16. A composition comprising a therapeutically effective amount of at least one osmolyte selected from a group consisting of betaine, taurine and myo-inosiol, their salts and precursors.

17. A composition according to claim 16 capable of providing a plasma concentration of about 0.01 to 10 mM of osmolytes.

18. A method of treating or preventing the effects of an infection, an inflammation or an immune dysfunction in a patient **characterized by** a supplying an effective amount of at least one osmolyte.
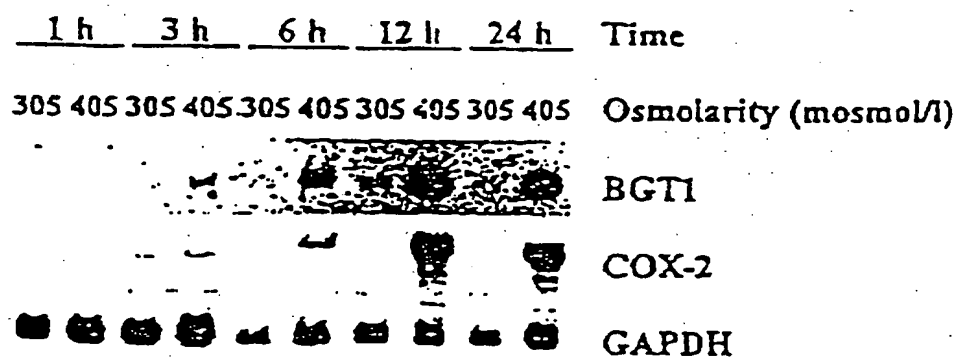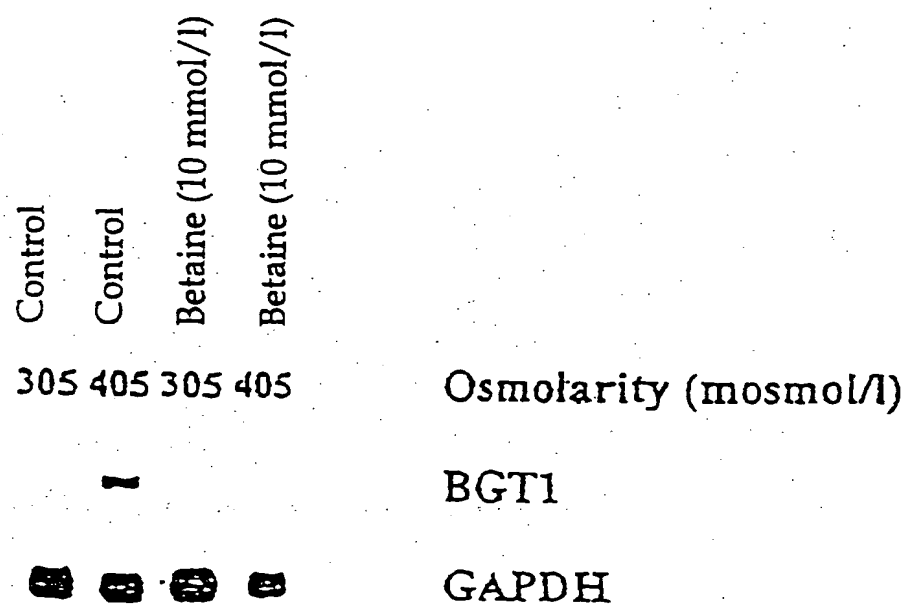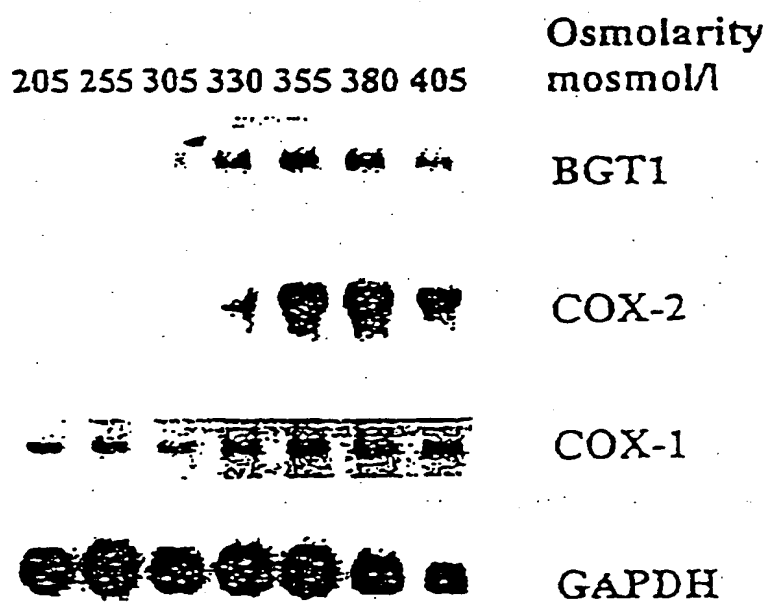
1 h		3 h		6 h		12 h		24 h		Time
305	405	305	405	305	405	305	405	305	405	Osmolarity (mosmol/l)
										BGT1
										COX-2
										GAPDH

Fig. 1

Fig. 2

205 255 305 330 355 380 405   Osmolarity
mosmol/l

BGT1

COX-2

COX-1

GAPDH

Fig. 3

control　Betaine (0,1 mmol/l),　Betaine (1 mmol/l)　Betaine (10 mmol/l)　Taurine (10 mmol/l)　Glutamine (10 mmol)

BGT1

GAPDH

Fig. 4

Fig. 5 A

Fig. 5 B

2 h	6 h	12 h	24 h	Time
305 405	305 405	305 405	305 405	Osmolarity (mosmol/l)

SMIT

TAUT

BGT1

GAPDH

Fig. 6

Fig. 7

Osmolarity
mosmol/l

205 305 405

SMIT

TAUT

BGT1

GAPDH
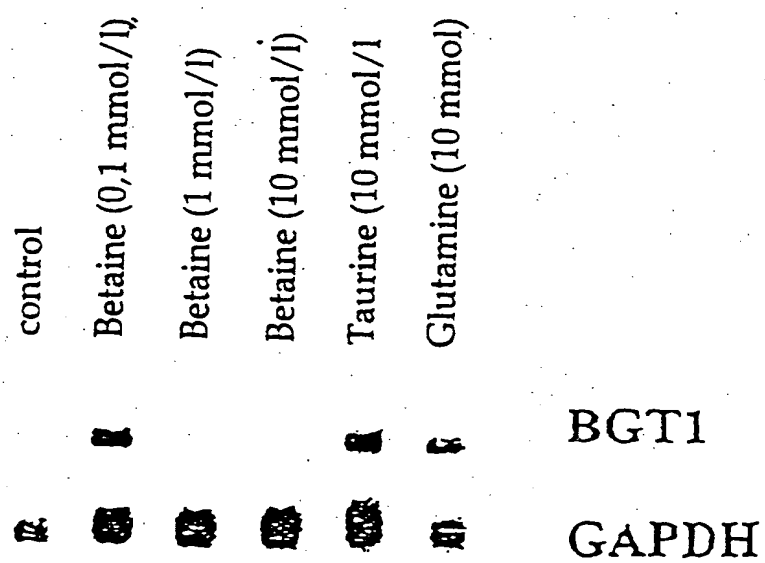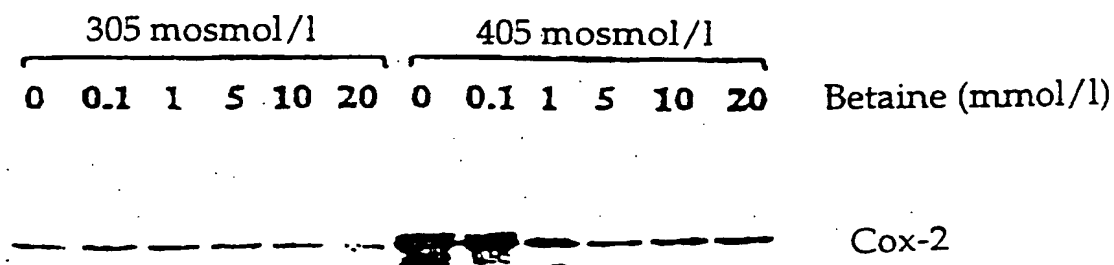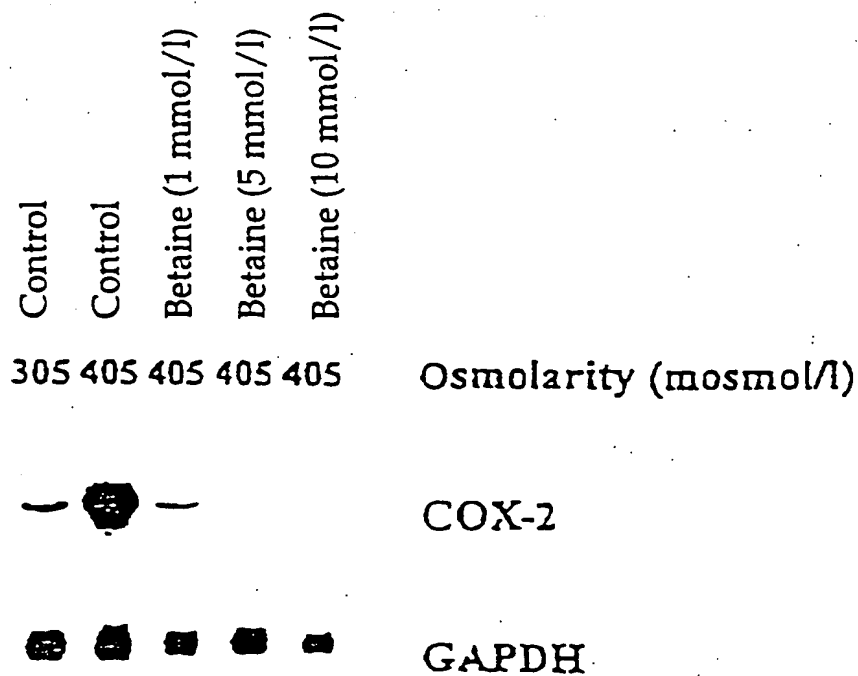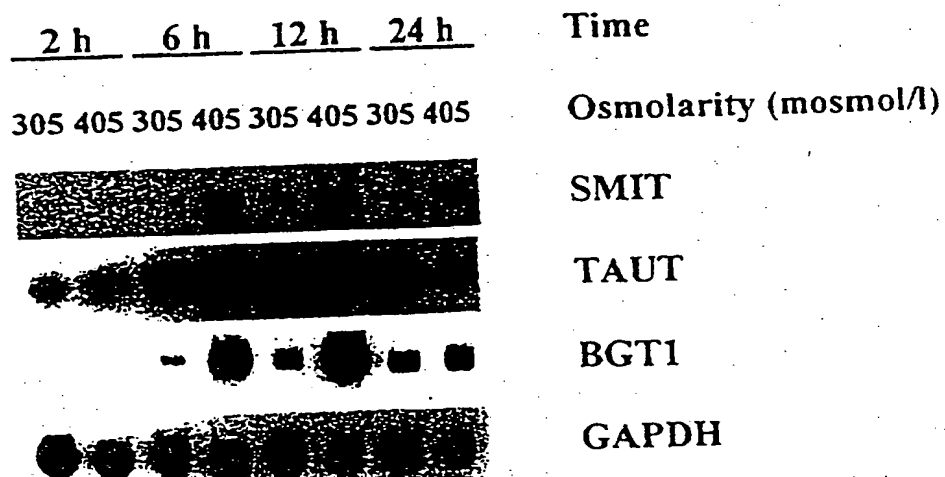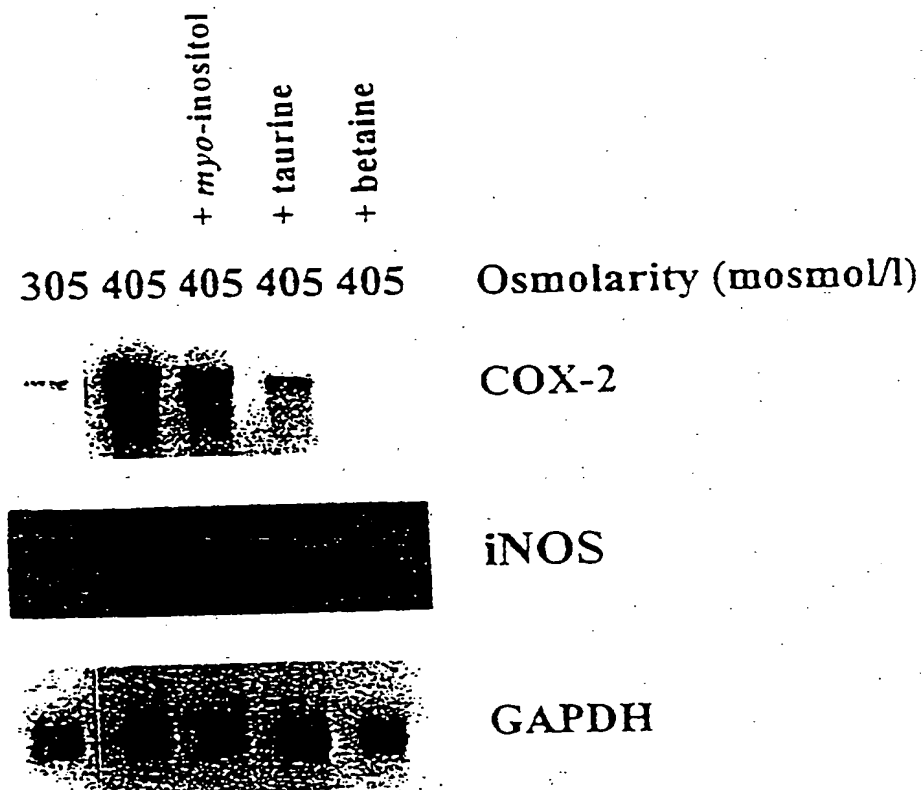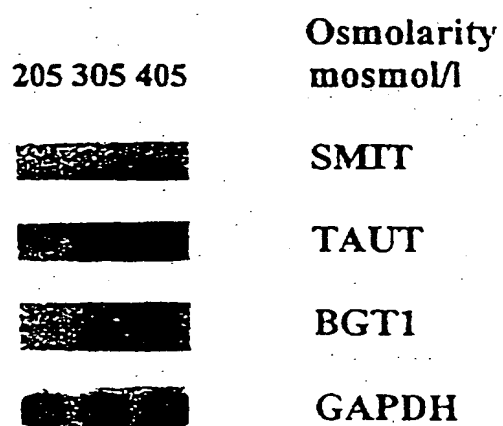
Fig. 8
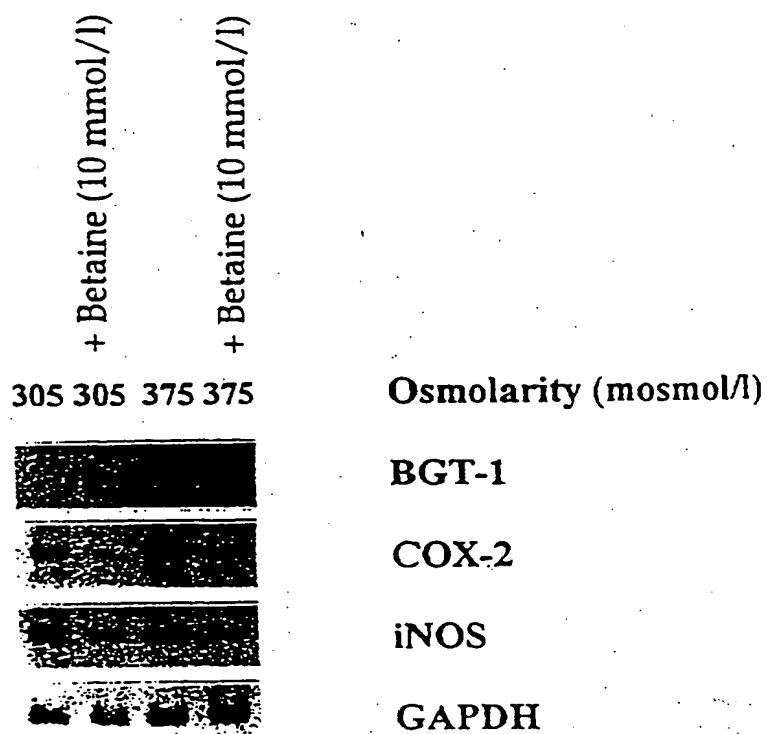
Fig. 9
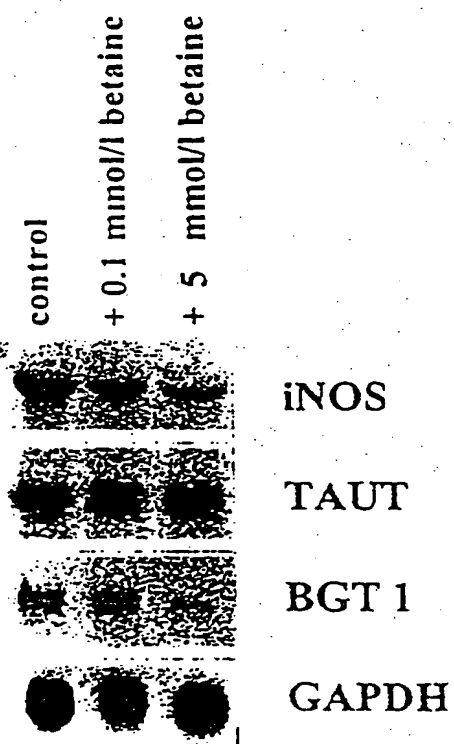
Fig. 10
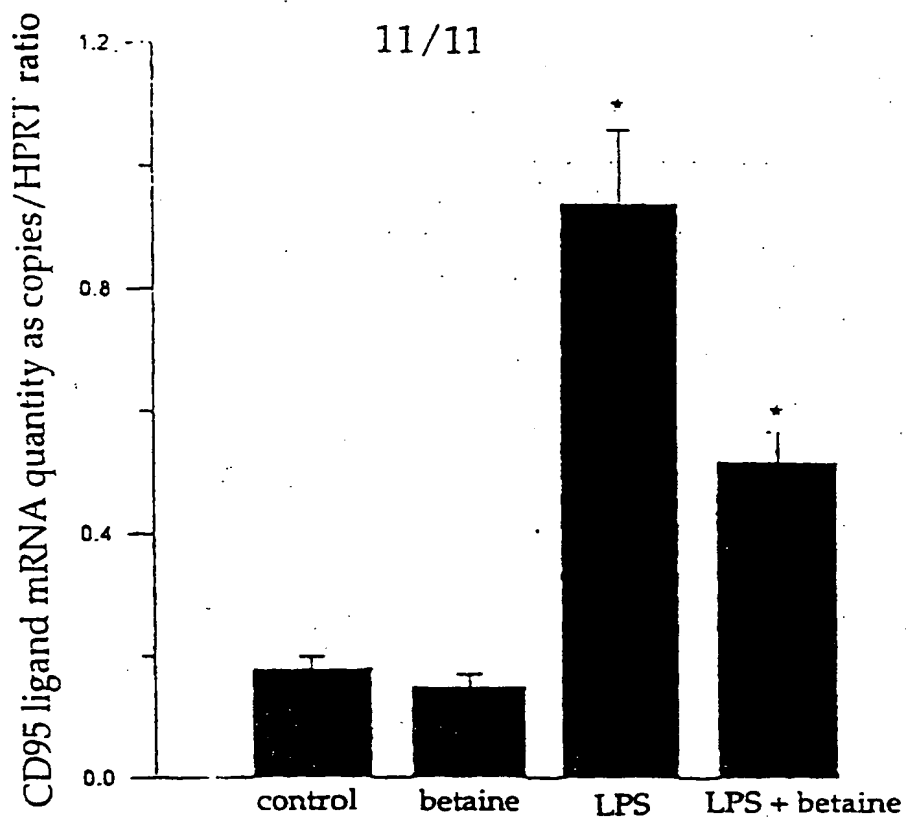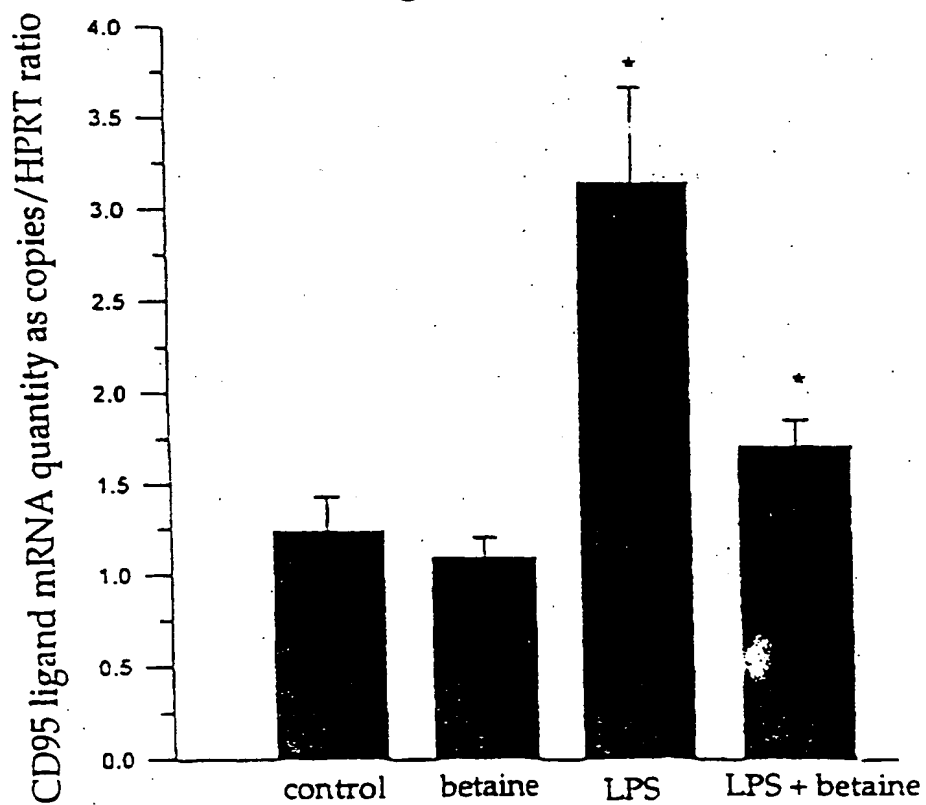
Fig. 11 A

Fig. 11 B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K31/205   A61K31/185   A61K31/045

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GASTROENTEROLOGY, vol. 110, no. 5, May 1996, pages 1543-1552, XP002036243 F. ZHANG ET AL.: "IDNETIFICATION OF BETAINE AS AN OSMOLYTE IN RAT LIVER MACROPHAGES (KUPFFER CELLS)" see the whole document ---	1-18
P,X	FEBS LETTERS, vol. 391, no. 3, August 1996, pages 293-296, XP002036244 F. ZHANG ET AL.: "MODULATION OF TUMOR NECROSIS FACTOR-alpha RELEASE BY ANISOOSMOLARITY AND BETAINE IN RAT LIVER MACROPHAGES (KUPFFER CELLS)" see the whole document ---	1-10, 16-18

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 July 1997	0 8. 08. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (- 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hoff, P

INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF LEUKOCYTE BIOLOGY, vol. 61, no. 2, February 1997, pages 161-166, XP002036245 E. PARK ET AL.: "PREACTIVATION EXPOSURE OF RAW 264.7 CELLS TO TAURINE CHLORAMINE ATTENUATES SUBSEQUENT PRODUCTION OF NITRIC OXIDE AND EXPRESSION OF iNOS mRNA" see the whole document ---	1-8,10, 16-18
P,X	US 5 516 798 A (FERKET PETER R) 14 May 1996 see the whole document ---	1-18
X	WO 91 14435 A (BRIGHAM & WOMENS HOSPITAL) 3 October 1991 cited in the application see abstract see page 12, line 5 - page 13, line 32 see page 19, line 13 - page 21, line 3 see page 22, line 10 - page 23, line 16; claims ---	1-18
X	THE JOURNAL OF IMMUNOLOGY, vol. 154, no. 9, 1995, pages 4778-4784, XP002036246 E. PARK ET AL.: "TAURINE CHLORAMINE INHIBITS PRODUCTION OF NITRIC OXIDE AND TNF-alpha IN ACTIVATED RAW 264.7 CELLS BY MECHANISM THAT INVOLVE TRANSCRIPTIONAL AND TRANSLATIONAL EVENTS" see the whole document ---	1-8,10, 16-18
X	JOURNAL OF LEUKOCYTE BIOLOGY, vol. 58, no. 6, 1995, pages 667-674, XP002036411 J. MARCINKIEWICZ ET AL.: "TAURINE CHLORAMINE, A PRODUCT OF ACTIVATED NEUTROPHILS, INHIBITS IN VITRO THE GENERATION OF NITRIC OXIDE AND OTHER MACROPHAGE INFLAMMATORY MEDIATORS" see the whole document ---	1-8,10, 16-18
X	EP 0 469 813 A (DERMA SYSTEMS LAB LIMITED) 5 February 1992 see abstract see page 3, line 2 - line 48; claims; examples ---	1-8,10, 16-18
X	US 3 728 444 A (COOK E ET AL) 17 April 1973 see the whole document ---	1-8, 10-14, 16-18

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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STN FILE SUPPLIER: BIOSIS; AN=51:132453, XP002036247 "TREATMENT BY A PREPARATION OF SOLUBLE ACIDIN OF RABIITS WITH EXPERIMENTAL WOUNDS INFECTED WITH STAPHYLOCOCCUS" & SB NAUCH RAB KIRG NAUCH-ISSLED INST TUBERK, vol. 6, 1969, pages 57-62, ---	1-9, 16-18
X	EP 0 659 349 A (SQUIBB BRISTOL MYERS CO) 28 June 1995 see abstract; claims; example 2 ---	11-14, 16,17
Y	BIOCHEMICAL JOURNAL, vol. 312, no. 1, 1995, pages 135-143, XP002036248 F. ZHANG ET AL.: "HYPEROSMOLARITY STIMULATES PROSTAGLANDIN SYNTHESIS AND CYCLOOXYGENASE-2 EXPRESSION IN ACTIVATED RAT LIVER MACROPHAGES" cited in the application see the whole document ---	1-18
Y	FEBS LETTERS, vol. 377, no. 1, 1995, pages 47-50, XP002036249 U. WARSKULAT ET AL.: "BETAINE IS AN OSMOLYTE IN RAW 264.7 MOUSE MACROPHAGES" cited in the application see the whole document -----	1-18

2

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/210

Remark : Although claim 18 is directed to a method of treatment of the
human/animal body the search has been carried out and based on
the alleged effects of the compound/composition.

OBSCURITIES:

In view of the large number of compounds which are theoretically contained
within the definition "osmolyte" of claims 1, 11, 18 the search had to be
restricted on economic grounds to the compounds specifically mentioned in
the description/examples (Art.6 PCT; Guidelines Part B, Chapt.II.7 last
sentence and Chapt.III, 3.7)

Claims searched completely: 7, 9, 10, 13, 15-17.
Claims searched incompletely: 1-6, 8, 11, 12, 14, 18.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5516798 A	14-05-96	NONE	
WO 9114435 A	03-10-91	AU 7584891 A US 5182299 A	21-10-91 26-01-93
EP 0469813 A	05-02-92	AU 641529 B AU 8133291 A CA 2048068 A JP 5097666 A US 5248680 A	23-09-93 13-02-92 31-01-92 20-04-93 28-09-93
US 3728444 A	17-04-73	US 3899589 A	12-08-75
EP 0659349 A	28-06-95	AU 8163394 A CA 2137431 A JP 7223939 A	29-06-95 23-06-95 22-08-95